The effect of a commercially available blueberry extract on tolerance to ischemia

An experimental study in rats

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ABSTRACT

Introduction: It has been suggested that extracts from grapes or berries such as blueberries are beneficial and protective against cardiovascular diseases, probably due to a high content of polyphenols like anthocyanins. Anthocyanins have several effects, for instance, being antioxidants and stimulate nitric oxide production. They are water-soluble, low-molecular size substances (molecular weight 4-500) and thus easily transverse cell membranes.

Hypothesis: Pre-treatment with a blueberry extract containing 80% anthocyanins protects against ischemia-reperfusion injury.

Methods: Rats were randomized into 4 groups and pretreated 2-3 weeks with blueberry extracts (Medox®, Medpalett, Sandnes, Norway) dissolved in their drinking water (approximately 0.7, 7, or 70 mg daily). Controls drank regular tap water. The hearts were harvested and retrogradely perfused, undergoing 30 minutes of global ischemia and 120 minutes of reperfusion. Infarct size as well as left ventricular pressures, coronary flow, and heart rate were measured.

Results: All three groups receiving blueberry extract had lower heart rate during reperfusion ($p=0.035$). There was no difference between groups in cardiac function or infarct size (the group with the lowest extract dose had a non-important reduction of infarct size, $p=0.27$).

Conclusion: The blueberry extract reduced heart rate during reperfusion, but had no important effect towards ischemia-reperfusion injury of the isolated rat heart.

BACKGROUND

Epidemiologic studies suggest that diets rich in plant foods protect against cancer and cardiovascular diseases. Plant foods contain fiber, vitamins, sulfur compounds, carotenoids, and organic acids, which contribute to the health effects, but they also contain a variety of polyphenols, which are increasingly regarded as effective protective agents. Polyphenols represent a wide variety of compounds, which are divided into several classes, e.g.

hydroxybenzoic acids, hydroxycinnamic acids, anthocyanins, proanthocyanidins, flavonols, flavones, flavanols, flavanones, isoflavones, stilbenes, and lignans.

The so-called "French paradox" is a term describing the fact that the incidence of ischemic heart disease is relatively low in France despite the tradionally high intake of saturated fat (Belleville et al 2002).This has been attributed to both a protective effect of ethanol as well as to the content of polyphenolic compounds such as proanthocyanidins and anthocyanins (Bagchi et al 2003, Amorini et al 2003, Serraino et al 2003). Grape extracts have in several studies been shown to be protective against ischemia and reperfusion injury (Sato et al 2001, Bagchi et al 2003). Furthermore these substances has also been claimed to be powerful antioxidants (Amorini et al 2003), have both vascular relaxant effects, as well as to be protective agents against atherosclerosis (dell'Agli et al 2004). Polyphenols are particularly abundant in red wine. Although anthocyanins are the most probable candidates of the protective effects, the different plants, fruits and berries – including grapes and blueberries - also contain a large number of other polyphenols, which may have an important role.

Recently it has been shown that anthocyanins are present in large quantities in blueberries and in an extract of blueberries (Medox®) made commercially available by a Norwegian company (Medpalett). This company has also patented the synthesis of the two most important anthocyanins (cyanidin and delphinidin) which represent more than 80 per cent of the total amount of polyphenols in blueberries.

ANTHOCYANINS

Anthocyanins area subclass of polyphenols found in food of vegetal origin. Anthocyanins give the intense colour to many fruits and berries. In contrast to the other flavonoids, anthocyanins carry a positive charge in the central ring structure and are thus cations. The number and nature of the different attached sugar molecules are responsible for the high number of anthocyanins, more than 500. They have been suggested to have beneficial effects in different disease states, such as for instance:

1. Cardiovascular disease due to anti-inflammatory and chemoprotective properties of the anthocyanins, because of scavenging reactive oxygen species and stimulation of endothelial nitric oxide synthase (eNOS) (Xu et al 2004) producing a more relaxed endothelial state.
2. They have also been found in experimental studies to be able to prevent circulatory failure and multiple organ dysfunction caused by endotoxin infusion (Sauebin et al 2004).

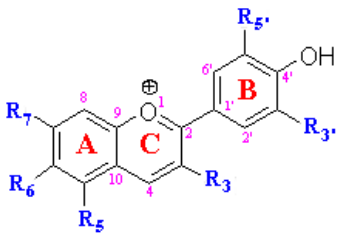


Figure 1. Basic structure of an anthocyanin without sugar group.

Medox:

The content of the Medox capsules are mostly the anthocyanins cyanidin and delphinidin. They are mainly present with the sugar molecules of glucose and galactose. There is also maltodextrin in the capsules, approximately contributing to 20 % of the weight of the capsule. The information about Medox was provided by the company Medpalett.

HYPOTHESES

1. Pretreatment with Medox® is protective against cardiac ischemia-reperfusion injury.

METHODS

I. Pretreatment

Different concentrations of blueberry extracts in the drinking water of rats. The animals were stalled alone in cages on shelves with regulated room temperature (21'C) and air humidity (55-60%), 12 hours of light and 12 hours of darkness. The animals were split into 4 groups, 3 groups drinking ad libitum in the form of blueberry extracts Medox® in 3 different concentrations. The control group will drink regular tap water. They will be fed with RM 3 (Scanbur BK AS, Nittedal), cages will be changed with BK bedding from Scanbur BK AS. We checked the rats daily. This feeding regime was used for 2 weeks before experiments. However, according to the progression of the experiments the exact number of feeding days varied a little (see below).

II. Langendorff perfusion

All rats were anesthetized intraperitoneally with pentobarbital sodium (100 mg/kg).
After thoracotomy, the heart was rapidly excised, placed in a temperature-regulated heart chamber, and Langendorff-perfused with gassed (5% CO_2, 95% O_2 at 37 C^o) Krebs Henseleit buffer ((mM/L): NaCl 118.5, $NaHCO_3$ 25, KCl 4.7, KH_2PO_4 1.2, $MgSO_4/7H_2O$ 1.2, Glucose/H2O 11.1, $CaCl_2$ 1.8)(Langendorrf et al 1897). The perfusion pressure was kept constant at 70 mmHg. Isovolumetric recording of left ventricular systolic (LVSP) and end-diastolic (LVEDP) pressures were obtained by a balloon inserted into the left ventricle through the left atrium. LVEDP was set to 5-10 mmHg at the end of the stabilization period (20 min.) and then the volume of the balloon was kept unchanged throughout the experiment. Coronary flow (CF) was measured by timed collections (at baseline, and after reperfusion of 30, 60, 120 min) of the coronary effluent. Left ventricular developed pressure was calculated as LVDP = LVSP – LVEDP. Heart rate (HR) and arrhythmias were evaluated from the pressure curves. We had the following inclusion criteria: LVdevP >100 mmHg, HR>220-360 beats/min, CF 8-20 ml/min, LVEDP 5-10mmHg. Hearts with irreversible arrhythmias (defined as either asystolia or ventricular fibrillation lasting more then 30 minutes) during reperfusion were excluded.

Global ischemia was induced by stopping perfusion. The following protocol was used: After 20 minutes of stabilization, the hearts were exposed to 30 minutes of global ischemia followed by reperfusion for 120 minutes, when infarct size was evaluated (Figure 2).

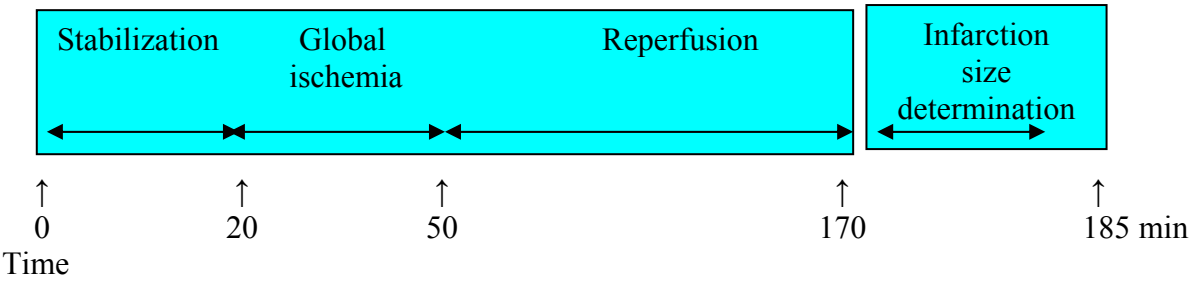


Figure 2.
Scheme of experimental protocol.

The rats were randomized and divided into the following groups (n = 10);

1. Control group drinking regular tap water.
2. 70 mg blueberry extract in the drinking water daily.
3. 7 mg blueberry extract in the drinking water daily.
4. 0,7 mg blueberry extract in the drinking water daily.

III. Measurements

1. Cardiac function:

LV pressure (LVSP, LVEDP), HR, CF (ml/min) was measure at the following time points: After Stabilization and during perfusion for 5, 10, 20, 30, 60, 90 and 120 minutes.

2. Arrhythmia Rate:

Irreversible arrhythmias (% of hearts)

3. Infarction size:

At the end of experiments, the heart was trimmed to remove the atrium, the right ventricular free wall, and connective tissues and was sliced transversely in a plane perpendicular to the apical-basal axis into approximately 1-mm-thick slices. The slices were immersed in phosphate-buffered saline containing 2% triphenyltetrazolium chloride for 15 min at 37°C. The brick red area was traced with the use of National Institutes of Health 1.61 image-processing software (Bethesda, MD), and each digitized image was subjected to equivalent degrees of background subtraction, brightness, and contrast enhancement for improved clarity and distinction. The areas at risk (equivalent to total LV mass), as well as the infarct zones of each slice, were calculated by the use of a computer program.

4. Statistics

Data are presented as individual values and mean value, or as mean and SEM for continuous data. Statistics are either analysis of variance repeated measures (for continuous data) or Students t-test with Bonferroni correction

RESULTS

Figure 3
There was no difference infarct size between the groups. Group 1 is control, groups 2-4 were daily fed with blueberry extract 70, 7, and 0.7 mg respectively.

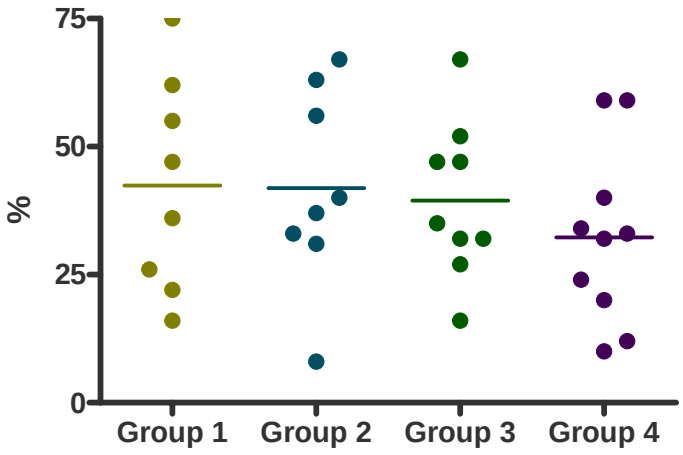


Figure 4
There was no difference in LVdevP between the groups. Group 1 is control, groups 2-4 were daily fed with blueberry extract 70, 7, and 0.7 mg respectively.

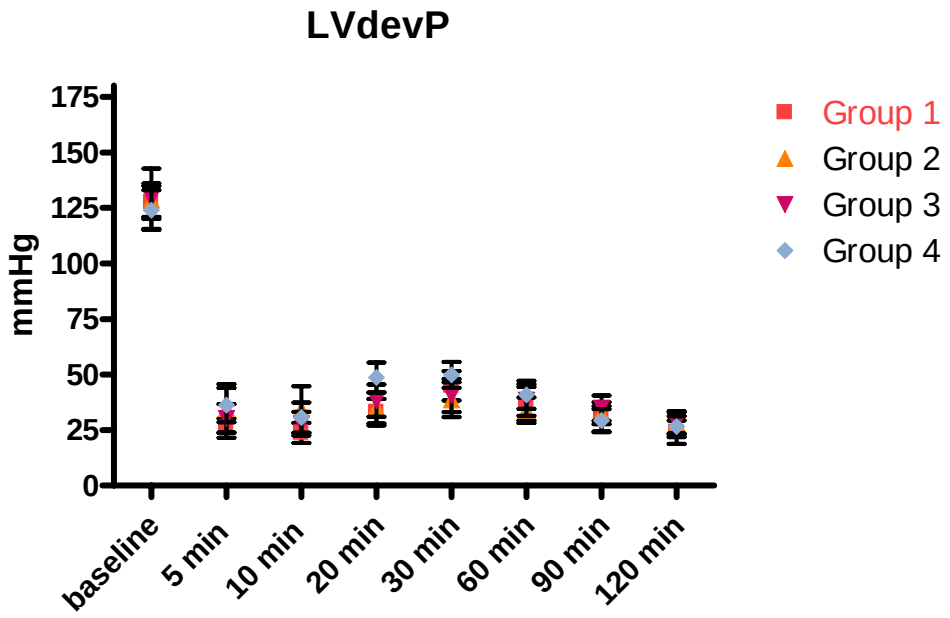


Figure 5
There was no difference in LVEDP between the groups. Group 1 is control, groups 2-4 were daily fed with blueberry extract 70, 7, and 0.7 mg respectively.

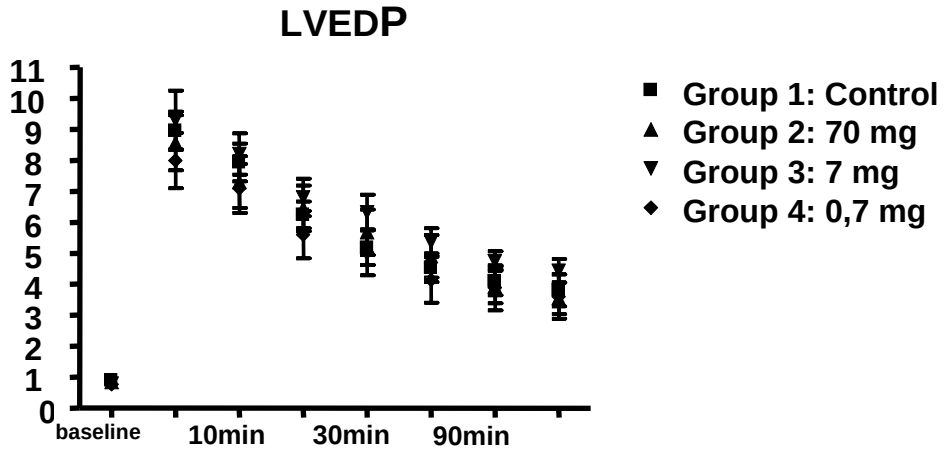


Figure 6
There was no difference in CF between the groups. Group 1 is control, groups 2-4 were daily fed with blueberry extract 70, 7, and 0.7 mg respectively.

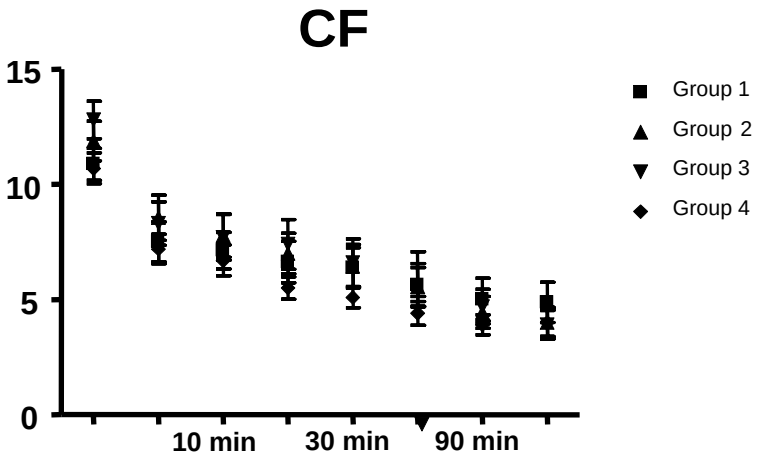


Figure 7
There were no important differences between weight of the rats between the groups. Group 1 is control, groups 2-4 were daily fed with blueberry extract 70, 7, and 0.7 mg respectively.

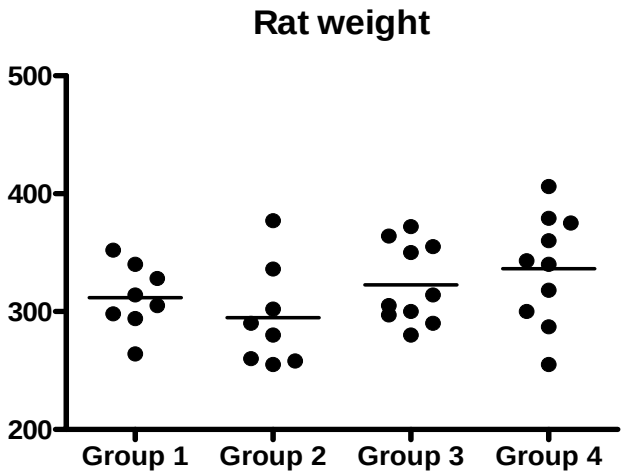


Figure 8
There was no important difference the number of feeding days between the groups. Group 1 is control, groups 2-4 were daily fed with blueberry extract 70, 7, and 0.7 mg respectively.

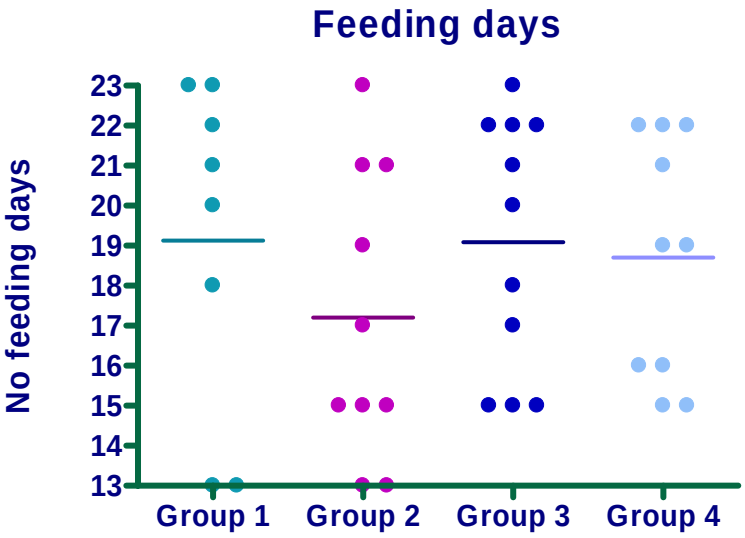


Figure 9
The number of feeding days had no effect on infarct size (IA). Group 1 is control, groups 2-4 were daily fed with blueberry extract 70, 7, and 0.7 mg respectively.

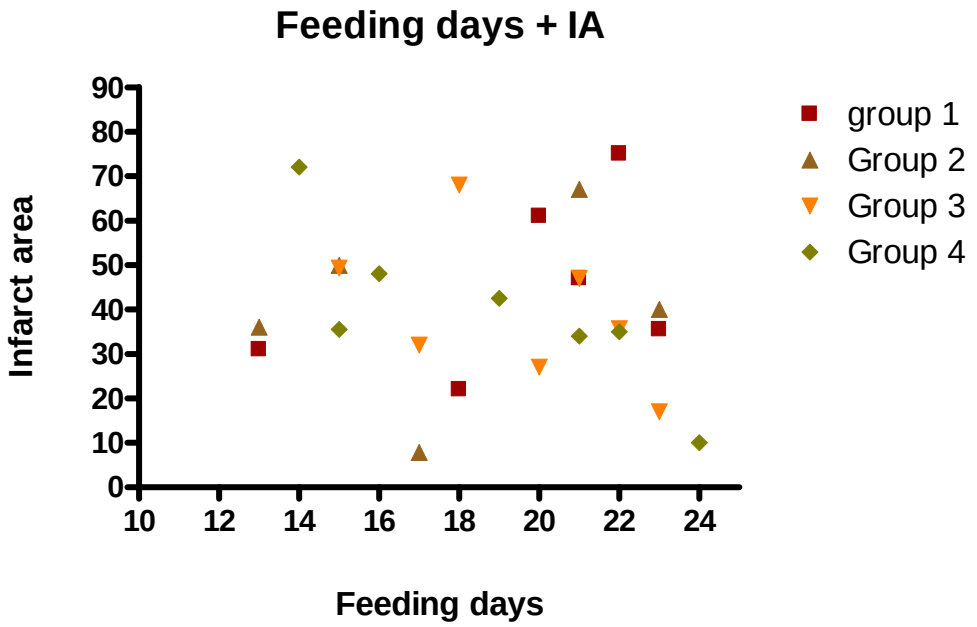
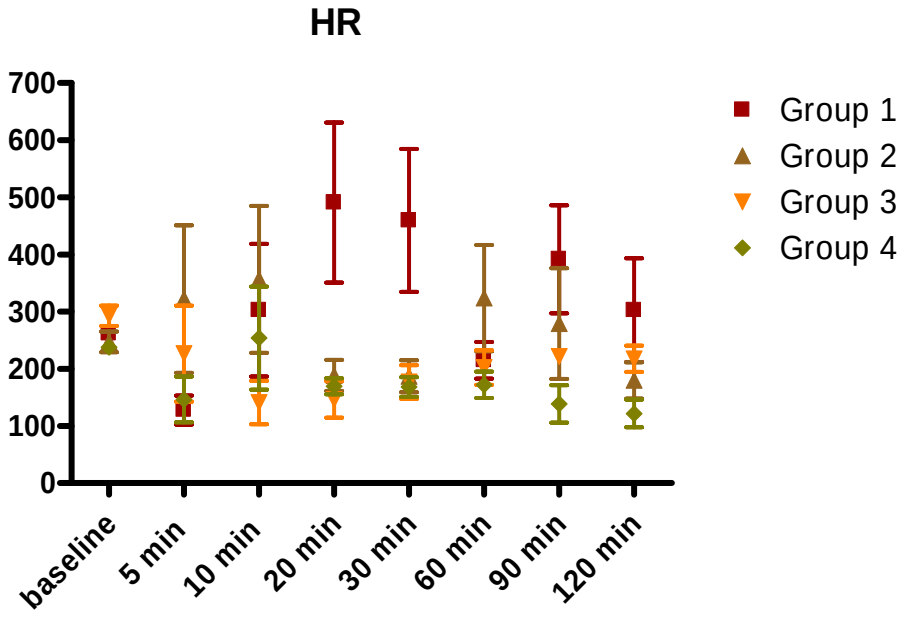


Figure 10
All groups given anthocyanins had occurrence of reduced fibrillation. This is most clear in the time points after 60 minutes of reperfusion. Group 1 is control, groups 2-4 were daily fed with blueberry extract 70, 7, and 0.7 mg respectively.



DISCUSSION

In the present investigation no pretreatment with various amounts of Medox®, 80% of which is anthocyanins, increased tolerance to ischemia in the isolated perfused rat heart. This lack of protection was evident as neither reduction of infarct size nor any attentuation of postischemic cardiac dysfunction. However, there was one effect on postischemic heart function: all hearts from rats pretreated with Medox® had reduced heart rate during reperfusion. This effect was independent on the amount of Medox® in the drinking water. Furthermore, there was a slight tendency towards a reduced infarct size in the group given the lowest amount of anthocyanine.

This is in disagreement with series of studies which have suggested that anthocyanins are cardioprotective. Amorini et al (2003) found that cyanidin protected the isolated rat hearts against exogenously induced oxidative injury. Concentrations of 10 or 30 μM cyanidin were used. Shin et al (2006) pretreated rats with peroral Medox® 75 mg twice during the last 24 hours experiments, and they found a significant reduction of brain infarct after occlusion and reperfusion of the middle cerebral artery. In another study anthocyanins were extracted from soybeans and rats were pretreated with 25, 50 or 100 mg/kg anthocyanins perorally 24 hours before the heart were harvested and perfused (Kim et al 2006). This pretreatment protected the hearts against ischemia-reperfusion injury.

We have no clear explanation why our findings are in discrepancy with these studies. It is of course important to know whether the our rats really had intake of anthocyanins. When staying in the drinking water for 24 hours less than 10 per cent of anthocyanins were inactivated (personal communication from Medpalett). Furthermore, the rats drank the water with anthocyanins as shown in the amount of water consumed.

Maybe the concentrations we used were too high and therefore were toxic. This may be indicated since there is a tendency in the group with the lowest concentration to have reduced infarct size. The other groups could have too high concentrations of anthocyanins, being toxic and thus preventing any protective effect the anthocyanins may have. It would therefore be interesting to experiment with even lower concentrations of anthocyanins to see whether even lower concentrations could induce more significant results.

Using anthocyanins from soybean coat significantly reduces infarct size by ischemia-reperfusion injury in an in vivo model(Kim et al 2006). Kim shows that anthocyanins dose-dependently decreased infarct size when given orally. However, they administered their anthocyanins 24 hours before the experiments. Maybe the anthocyanins only have an effect directly after ingestion. However, this does not seem likely since our rats were fed continuously and must have taken in some of the anthocyanins within the last 24 hours, though maybe not enough. Kim shows that infarct size seems to be decreased dose-dependently, but his highest concentration is far lower than our highest concentration (100 mg/kg vs. 280 mg/kg). Kim et al (2006) also used doses which are in between our other groups and showed reduced infarct size. There might be a difference between an in vivo model (Kim et al 2006) and our in vitro study. Kim et al (2006) used soybean coat anthocyanins containing delphinidin, cyanidine and petunidine. Medox® contains negligible amounts of petunidine, the protective effect of which might be underestimated. Or maybe something else in the soybean coat exerted the cardiac protective effect. Interestingly in a study published september 2006 it was shown that the flesh and skin of grapes had equally

protective effects in isolated, perfused rat hearts (Falchi et al 2006). These rats were fed for 30 days with either skin or grape flesh at an amount of 2.5 mg/kg body weight. This is comparable to our lowest dose of Medox® where a weak tendency towards a protective effect was observed. However, other factors might be playing a decisive effect in the study of Falchi et al (2006), because grape flesh does not contain anthocyanins. Still it was shown that flesh and skin had similar scavenging effects against reactive oxygen species (Falchi et al 2006).

There was in our study a lower heart rate during parts of the reperfusion in all groups given anthocyanins. No other study has investigated heart function using anthocyanins and thus we have little to compare our results to.

We are currently investigating effects of pure anthocyanins in rats in vivo to see if this can give us additional information concerning whether anthocyanins are cardio protective and to see if we can duplicate the study done by Kim. This project was however ment to be a pilot project to see if there was any indication of anthocyanins being cardioprotective. The results we have gotten so far leaves us somewhat in a difficult situation. This is because there is in fact an indication that there seems to be cardioprotective effect, but not as much as we had wanted. However, we have decided that we will continue investigating more closely the effect of the anthocyanins. We will now use a pure synthesized anthocyanine, this time introducing them in vivo into rats. The concentrations this time will also be lowered, so we will be able to see if our concentrations in deed were toxic.

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